

# Bone Sialoprotein Peptides Are Potent Inhibitors of Breast Cancer Cell Adhesion to Bone<sup>1</sup>

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## ABSTRACT

Bone and bone marrow are important sites of metastasis formation in breast cancer. Extracellular matrix proteins with attachment properties are generally believed to play a key role in tumorigenesis and metastasis formation. We have investigated whether mammary carcinoma cells (MDA-MB-231) can recognize constructs of the fairly bone-specific human bone sialoprotein, which encompass the RGD sequence (EPRGD-NYR). Exogenously added bone sialoprotein peptides with this amino acid sequence in their backbone structure, but not the more common fibronectin-derived GRGDS peptide, strongly inhibited breast cancer cell adhesion to extracellular bone matrix at micromolar concentrations. Most cyclic derivatives with the EPRGDNYR sequence were more effective inhibitors of tumor cell adhesion to bone than their linear equivalents. Furthermore, changes in the RGD-tripeptide of the backbone structure of the constructs, removal of the NYR flanking sequence, or a different tertiary cyclic structure significantly decreased their inhibitory potencies. In addition, the RGE-analogue EPRGENYR was capable of inhibiting breast cancer cell adhesion to bone, albeit to a lesser extent.

We conclude, therefore, that the inhibitory potency of the bone sialoprotein-derived peptides on breast cancer cell adhesion to bone is not solely due to a properly positioned RGD-motif alone but is also determined by its flanking regions, together with the tertiary structure of the EPRGDNYR peptide. Synthetic cyclic constructs with the EPRGDNYR sequence may, therefore, be potentially useful as antiadhesive agents for cancer cells to bone *in vivo*.

## INTRODUCTION

Breast cancer metastasizes frequently to the skeleton, leading to serious clinical complications such as bone pain, fractures, spinal cord compression, and hypercalcemia (1-4). The mechanisms underlying the apparent preference of mammary carcinoma cells for bone remain, however, poorly understood. The interactions of tumor cells with the bone microenvironment, together with the anatomical properties of the skeleton, are generally believed to be decisive during the final steps in the metastatic cascade, leading to the establishment and invasive growth of skeletal metastases (5-10).

The organic matrix of bone is composed of collagenous proteins (type I collagen fibers) representing 85-90% of total bone protein, whereas noncollagenous proteins comprise the remaining 10-15% (11-13). The list of proteins that can be isolated from mineralized bone matrix with cell attachment activity (adhesion proteins) is best headed by the proteins that contain RGD (Arg-Gly-Asp) motifs, a cell attachment recognition consensus sequence for several adhesion receptors of the integrin family (14, 15). Of the six identified RGD-containing extracellular bone matrix proteins (11, 13, 16, 17), fi-

bronectin, type I collagen, vitronectin, thrombospondin, OPN,<sup>3</sup> and BSP, only the last is almost exclusively restricted to bone tissue (18). This sialic acid-rich glycoprotein and its mRNA are found predominantly in the mature, matrix-producing osteoblasts and osteocytes (18-24). The BSP gene shows a distinct expression pattern in bone (23-25). In addition, epiphyseal cartilage cells like hypertrophic chondrocytes (18, 26) osteoclasts (18, 26, 27), and marrow stromal cells (28) also produce BSP. The human integrin-binding region of BSP has the more rare GRGDN rather than the more common GRGDS/T sequence.

Several studies have shown that extracellular matrix proteins, like BSP and OPN, may be involved in the recognition and adhesion of osteoclasts and their precursors to bone matrix, a process mediated through the vitronectin ( $\alpha_v\beta_3$ ) receptor (29-35). The  $\alpha_v\beta_3$  receptor is believed to play a key role in osteoclast formation (36) and osteoclastic resorption (29-36).

Besides a variety of functions in normal physiology, adhesion receptors in tumor cells appear to play important roles along the metastatic cascade of events leading to the establishment of distant metastases (9, 10, 37-41). Recently, we have shown that adhesion receptors of the  $\beta_1$  and  $\beta_3$  integrin family may be functionally involved in adhesion of mammary carcinoma cells to extracellular bone matrix (constituents; Refs. 42 and 43). Individual components of extracellular bone matrix may, therefore, be involved not only in the preferred bone homing of normal cells (like osteoclast precursors and osteoprogenitors) to bone but may also provide a fertile environment for breast cancer cells.

In this study, we examined the attachment characteristics of breast cancer cells to conformationally constrained peptide analogues designed to mimic the cell attachment site of human BSP. Several constructs that encompass the BSP-derived RGD-cell attachment motif EPRGDNYR in linear and cyclical conformations were tested (44). In addition, the effects of these exogenously added BSP peptides on breast cancer cell adhesion to bone were studied in three *in vitro* models of extracellular bone matrix. Results presented in this paper provide evidence that several peptide analogues that are based on the (RGD-containing) cell attachment site of BSP are recognized by breast cancer cells and may be potentially useful as antiadhesive agents for cancer cells to bone.

## MATERIALS AND METHODS

**Breast Cancer Cell Lines.** The breast cancer cell line MDA-MB-231 was purchased from the American Type Culture Collection (Rockville, MD). This estrogen receptor-negative cell line was established from a single pleural effusion obtained from a 51-year-old white woman with poorly differentiated adenocarcinoma (45). MDA-MB-231 cells injected into the left heart ventricle of nude mice have been shown to form osteolytic metastases *in vivo* (8, 46).<sup>4</sup>

Cells were cultured in RPMI 1640 + 10% fetal bovine serum + penicillin/streptomycin (Life Technologies, Breda, the Netherlands) in a humidified

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<sup>3</sup> The abbreviations used are: OPN, osteopontin; BSP, bone sialoprotein; SFCM, serum-free conditioned medium.

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incubator at 37°C at 5% CO<sub>2</sub> until confluency. For attachment assays, tumor cells were cultured until approximately 90% confluency and were dissociated into single-cell suspensions from the tissue culture flasks using 0.125% trypsin-0.05% EDTA in PBS for 3 min (see attachment assays).

**Synthetic Peptides, Proteins, and Reagents.** BSA was purchased from Miles Scientific, Inc. (Naperville, IL). SFCM was obtained from confluent normal, primary human trabecular bone cells incubated for 24 h in serum-free medium supplemented with 0.5% ITS<sup>+</sup> [insulin, transferrin, and selenium (2.5 µg/ml), 0.5 mg/ml BSA, and 0.5 µg/ml linoleic acid; Collaborative Research, Inc., Bedford, MA]. SFCM contains a mixture of various extracellular bone matrix components, including BSP, and reflects the diversity and composition of extracellular bone matrix *in vivo* as described earlier (11, 16, 47).

The linear and cyclic peptides used in this study were synthesized using an automated solid phase peptide synthesizer as described earlier (44). We have used three linear synthetic BSP peptides (BSA-BA6, BSA-LBP, and CP-3), five cyclic BSP peptides (BSA-CNB, CNB, CCP, Ac-CNB, and C-BC3; Fig. 1), and two unrelated peptides C-CB1 and GRGDS (Calbiochem, La Jolla, CA). C-CB1 was reported earlier to have an antithrombotic action by interfering with the heterodimeric glycoprotein integrin receptor GPIIb/IIIa (48). The GPIIb/IIIa receptor, like several members of the integrin family, contains a binding site for the tripeptide Arg-Gly-Asp (RGD).

BSA, GRADS, and GRGES were used as negative controls and did not affect adhesion of mammary carcinoma cells to the various substrates tested. For reasons of clarity, BSA, GRGES, and GRADS controls are not depicted in

the figures of this report. To study the importance of the RGD adhesion recognition sequence and the relative contribution of RGD flanking regions in CNB, the tripeptide sequence was replaced by RGE, resulting in the peptide CCP.

**Cell Attachment Assays: Proteins Coated onto Plastic.** Cell attachment assays were performed in bacteriological 96-well plates dotted with glycoproteins, as described previously (16). In brief, 10 µl of SFCM or peptide construct in PBS containing 1 mM Ca<sup>2+</sup> was applied, resulting in a protein-coated area ("dot") of 0.12 cm<sup>2</sup>. After 16 h at 4°C, the fluid was removed, and 100 µl of 60% methanol were added to each well for 2 h at 4°C. The methanol fixation was found to improve the stability and durability of the protein-coated area without altering the biological activity of the substrate (16). The methanol was removed, and wells were washed for 30 min at 4°C with washing buffer [50 mM Tris-HCl (pH 7.8), 110 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.1 mM phenylmethylsulfonyl fluoride, 1% BSA, and 0.1 µM sodium azide] to block unbound sites on the plastic. After removal of the washing buffer, the wells were washed three times with serum-free medium (RPMI 1640) supplemented with glutamine and 0.5% ITS<sup>+</sup>.

Cells were preincubated in RPMI 1640 (+ HEPES) + 10% fetal bovine serum + penicillin/streptomycin in a humidified incubator at 37°C at 5% CO<sub>2</sub> until 90% confluency. For attachment assays, tumor cells were dissociated from the flasks using 0.125% trypsin-0.05% EDTA solution in PBS (pH 7.2) for 3 min. Cells were washed and resuspended in serum-free medium (RPMI 1640 containing 0.5% ITS<sup>+</sup>) and seeded at a density of 10,000/cm<sup>2</sup> (2800 cells/150 µl) on the matrix coated 96 wells. The 96-well dishes were incubated at 37°C at 5% CO<sub>2</sub> for a maximum of 3 h. Each well was washed three times with serum-free medium, and attached cells were fixed for 20 min with 80% methanol at 4°C and stained with AmidoBlack. Two nonoverlapping microscopic fields within each protein-coated area were counted. Each group was performed in 4-fold, and experiments were repeated three times.

The adhesion of mammary carcinoma cells to constructs coated onto plastic was determined at various concentrations. Coating efficiencies to bacteriological plastic differed between the tested constructs. Significantly detectable coating was obtained only with BSP-derived peptides conjugated to BSA (maximal coating efficiencies about 65% at 1 µM RGD content), whereas coating efficiencies of other peptides were low (<10%) or nondetectable at various concentrations tested (0.01–10 µM). At concentrations higher than 1.0 µM, the absolute amount of plastic bound peptides (BSA-CNB, BSA-BA6, and BSA-LBP) remained constant (results not shown).

**Adhesion of Breast Cancer Cells to Bovine Cortical Bone Slices.** Bovine cortical bone slices were a kind gift from Dr. Tiina Laitala (University of Oulu, Oulu, Finland). Tissue culture plates (24 wells) were coated with 500 µl of 1% agarose in PBS (pH 7.2) to prevent non specific adhesion to the tissue culture plastic. After the agarose cooled down and became solid, a drop of melted agarose was added to each well on the agarose layer, and the bone slices were placed on top of this drop, resulting in a bone slice fixed to the agarose underlay. Subsequently, the wells were washed twice with 1 ml of serum-free culture medium and preincubated for 30 min. Tumor cells were seeded on top of these slices at a density of 100,000 cells/500 µl and incubated for 3 h. After incubation, the bone slices were removed from the wells and washed three times in PBS and fixed for 30 min with 500 µl 3% paraformaldehyde in PBS containing 2% sucrose. Bone slices were stained for 8 min with 1% toluidine blue in 1% sodium borate and, after fixation, were washed three times with PBS and mounted on glass coverslips with Histomount (National Diagnostics-Brunswick Chemie, Amsterdam, the Netherlands). Stained bone slices were kept dark before histological examination. Cells located within two nonoverlapping microscopic fields were counted at the center of each bone slice. Each experimental group was performed in 4-fold, and experiments were repeated at least three times.

**Adhesion of Breast Cancer Cells to Frozen Sections of Developing Trabecular Bone.** Two-day-old neonatal mice (Swiss Albino) were killed, and their tails were dissected, stretched in Tissue Tek (Miles Scientific, Naperville, IL), frozen in liquid N<sub>2</sub> for at least 10 min, and stored at -80°C. Five-µm sections were cut at -20°C using a cryostat (Microm HM 500 M) and subsequently transferred to 3-aminopropyltriethoxy-silane (Sigma Chemical Co, St. Louis, MO)-coated glass coverslips and stored at -80°C until use.

Prior to adhesion experiments, the frozen sections were air dried at room temperature for 30 min. Around each tissue section, a (hydrophobic) line was drawn with a DAKO pen (DAKO A/S, Glostrup, Denmark) to avoid spreading

<u>Peptide designation</u>	<u>Peptide structure</u>
BSA-BA6	EPRGD-DPA-CM-BSA
BSA-LBP	EPRGDNYR-DPA-CM-BSA
CP3	EPRGENYR
BSA-CNB	BSA-CM-A-DPA-EPRGDNYRCysNH <sub>2</sub>
CNB	DPA-EPRGDNYRCysNH <sub>2</sub>
CCP	C-EPRGENYR-BPA
Ac-CNB	Ac-DPA-EPRGDNYRCysNH <sub>2</sub>
C-BC3	EPRGDNYRCysNH <sub>2</sub>
C-CB1	dYRGDCysNH <sub>2</sub>

Fig. 1. Designated abbreviations and structures of linear and cyclic peptides used in this study. Six cyclical (BSA-CNB, CNB, CCP, Ac-CNB, C-BC3, and C-CB1) and three linear (BSA-BA6, BSA-LBP, and CP3) were studied. Single letter codes are used for amino acids, except for cysteinamide (CysNH<sub>2</sub>). GRGDS was used as an RGD control. GRADS, GRGES, and BSA were used as negative control peptides/proteins. Ac, acetyl; DPA, α,β-L-diaminopropionic acid; BPA, N<sup>β</sup>-bromoacetyl-diaminopropionic acid; CM, S-carboxymethylcysteine.

of the cell suspension over the entire glass coverslip area. Subsequently, Tissue Tek was removed by incubating the sections for 5 min in a large volume of PBS. The sections were incubated at 4°C with 100  $\mu$ l of PBS + 2% w/v BSA-fraction V for 2 h to avoid nonspecific binding. Eighty thousand breast cancer cells in 80  $\mu$ l serum-free medium (RPMI 1640 + ITS<sup>+</sup>) were added per section in the presence or absence of 2  $\mu$ M synthetic peptides (Fig. 1) and incubated for 1 h at 37°C in a humidified incubator (5% CO<sub>2</sub>). After incubation, the sections were washed three times with PBS (to remove nonadherent cells), and attached cells were fixed in 4% paraformaldehyde for 30 min. Subsequently, the sections were washed with PBS and stained with hematoxylin for 1 min. The hematoxylin was removed, and the stained sections were washed in running tap water for 5 min. The sections were mounted in Histomount (National Diagnostics-Brunschwig Chemie) and covered with coverslips.

Cell numbers were determined by counting nonoverlapping microscopic fields for each central section of vertebra at an original  $\times 200$  magnification. The mean value of two microscopic fields within one vertebral section was determined within each section ( $n = 5$ ). The central sections of the tail were made from the same animal and used for control and experimental groups. Each experiment was repeated at least three times ( $n = 3$ ).

**Inhibition of Breast Cancer Cell Adhesion to Extracellular Bone Matrix by Synthetic Peptides.** Breast cancer cell attachment to bone matrix was determined in the continuous presence of synthetic peptides at various concentrations, and IC<sub>50</sub>s were calculated.

**Statistics.** Significances between experimental groups within each experiment were determined using a factorial one-way analysis of variance, followed by a Fisher's PLSD test.

## RESULTS

**Adhesion of Breast Cancer Cells to Varying Concentrations of Coated BSP Peptide Conjugates.** MDA-MB-231 cells were monitored for their ability to adhere to different concentrations of synthetic BSP peptides conjugated to BSA that were coated onto bacteriological plastic (96-well plates). Peptide structures are based on the RGD-dependent cell attachment site of human bone sialoprotein. Mammary carcinoma cells bound to immobilized BSP peptides and subsequently spread in a dose-dependent manner (Fig. 2). Maximal cellular adhesion of mammary carcinoma cells was obtained at peptide concentrations of 1  $\mu$ M and higher (Fig. 2). However, differences were found in the ability of MDA-MB-231 cells to adhere to the various peptides. BSA-CNB promoted cellular adhesion most strongly, followed by BSA-LBP and BSA-BA6 respectively. Fig. 3 depicts the morphological appearance of MDA-MB-231 cells following adhesion to SFCM

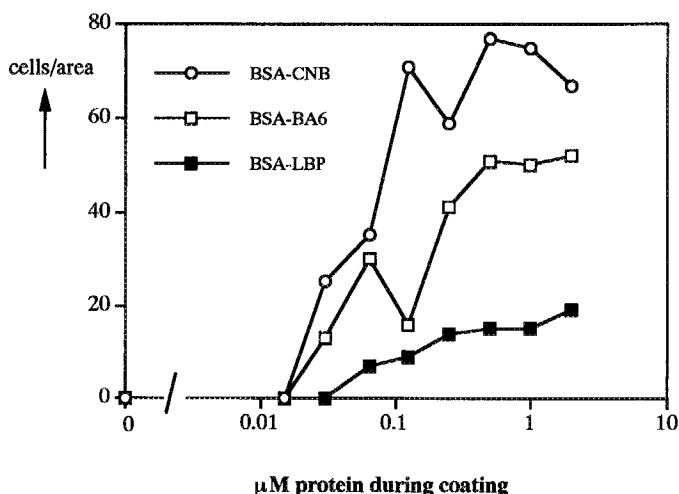


Fig. 2. Dose-dependent attachment patterns of breast cancer cells (MDA-MB-231) to the synthetic BSP-peptides BSA-CNB, BSA-BA6, and BSA-LBP that are coated onto plastic. A representative experiment is shown. The experiment was repeated three times.

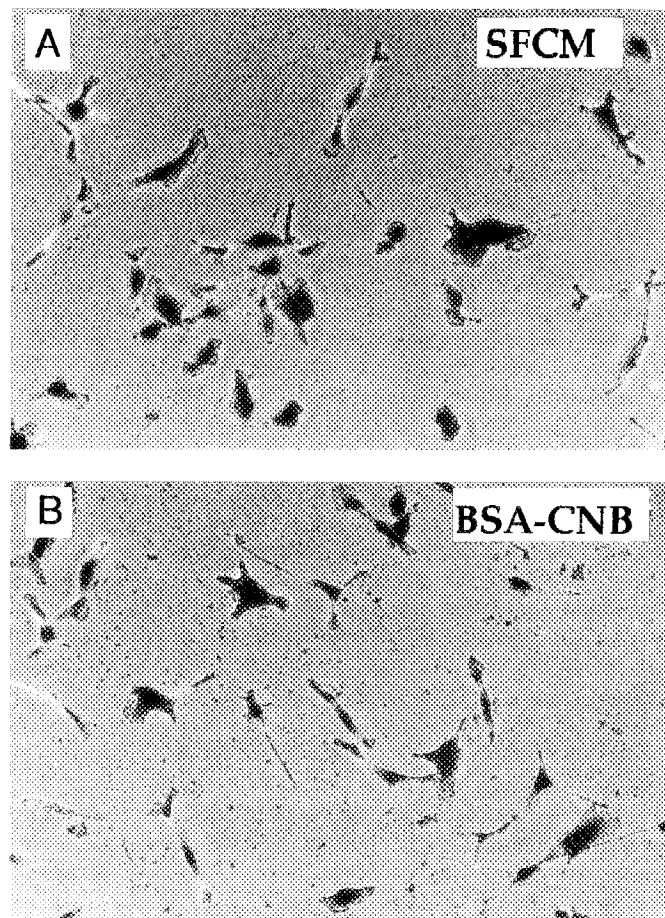


Fig. 3. The morphological appearance of MDA-MB-231 cells following adhesion to SFCM (A) and a cyclic EPRGDNYR construct conjugated to BSA, BSA-CNB (B). The wells of a microtiter plate were coated with 2  $\mu$ M peptide or 1:1 diluted SFCM in PBS containing 2 mM Ca<sup>2+</sup> (see "Materials and Methods").  $\times 200$ .

of normal human trabecular bone cells (Fig. 3A, positive control) and BSA-CNB (Fig. 3B) when coated onto plastic.

**Effects of Exogenously Added Peptides on Attachment of Breast Cancer Cells to Extracellular Bone Matrix.** Three *in vitro* attachment assays were used to investigate whether exogenously added BSP peptides can modulate the adhesion of breast cancer cells to extracellular bone matrix. MDA-MB-231 cells adhered strongly to immobilized SFCM of normal human trabecular bone cells. Exogenously added GRGDS peptide, at concentrations up to 300  $\mu$ M (IC<sub>50</sub> = 400  $\mu$ M; Table 1), did not significantly affect the attachment of MDA-MB-231 cells to SFCM.

In contrast to GRGDS, seven of eight tested synthetic BSP-peptides significantly inhibited adhesion to various degrees (Table 1). This is further illustrated in Fig. 4 with equimolar concentrations of the tested peptides (2  $\mu$ M). At this concentration, the cyclic peptides BSA-CNB and CNB were more potent inhibitors of adhesion ( $\pm 80\%$  inhibition) than the linear peptides BSA-LBP or BSA-BA6 with similar or shortened amino acid sequences (50 and 30%, respectively). Furthermore, blocking of the NH<sub>2</sub> terminus by the addition of an acetyl (Ac) group decreased the inhibitory potency of CNB.

A change in tertiary structure by modification of the ring size, leading to the smaller cyclic C-BC3 peptide with identical EPRGDNYR amino acid sequence, resulted in total loss of the inhibitory effect on the adhesion of breast cancer cells to bone matrix (Fig. 4; Table 1). Furthermore, replacement of the RGD tripeptide by RGE (CCP) significantly decreased the inhibitory potency of CNB (Fig. 4;

Table 1 Half-maximal inhibitory concentrations ( $IC_{50}$ s) of exogenously added peptides, constructs, and proteins during adhesion of MDA-MB-231 cells to SFCM of normal human trabecular bone cells

Peptide	$IC_{50}$ ( $\mu$ M) for cellular adhesion
RGD control peptide	
GRGDS	400
Negative controls	
BSA-V	>500
GRADS	>500
GRGES	>500
Linear peptides/constructs	
BSA-BA6	2-20
BSA-LBP	2-20
CP3	400
Cyclic peptides/constructs	
BSA-CNB	0.002-0.02
CNB	0.02-0.2
Ac-CNB	2
CCP	2-20
C-BC3	>500
C-CB1	>500

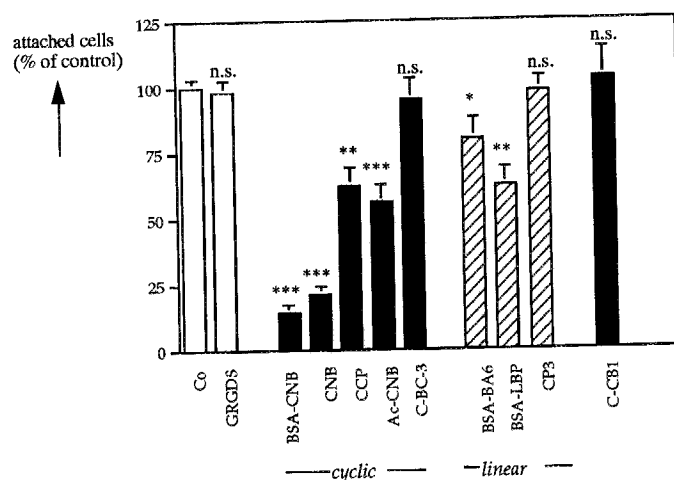


Fig. 4. Inhibitory effects of exogenously added BSP-peptides ( $2 \mu$ M) on adhesion of breast cancer cells (MDA-MB-231) to extracellular bone matrix (SFCM coated onto plastic). Two  $\mu$ M GRGDS were used to study the RGD-dependent nature of tumor cell attachment. BSA, GRADS, and GRGES were used as negative controls and did not change adhesion of mammary carcinoma cells to bone matrix (results not shown). Each group was performed in 4-fold, and experiments were repeated three times; bars, SEM. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.005$ ; \*\*\*,  $P \leq 0.0005$ .

Table 1). However, this RGE analogue of CNB was still capable of inhibiting breast cancer cell adhesion to bone, albeit to a lesser extent.

Removal of the NYR flanking sequence of the peptide BSA-LBP, resulting in BSA-BA6, significantly decreased its inhibitory potency on breast cancer cell attachment. In addition, a different (smaller) EPRGDNYR ring structure, resulting in C-BC3, also decreased the inhibitory potency of the cyclic CNB peptide (Fig. 4; Table 1). Cyclizing the RGD motif with non-BSP-specific flanking regions was ineffective (C-CB1; Fig. 4; Table 1).

Similarly, as shown in Fig. 5, breast cancer cell adhesion to bovine cortical bone slices was significantly inhibited by the tested cyclic and linear BSP-peptides (BSA-CNB, CNB, BSA-LBP, and BSA-BA6) but not with GRGDS or negative control peptides (GRADS, GRGES, and BSA; data not shown). In this assay as well, the cyclic BSP-peptides (BSA-CNB and CNB) were more effective inhibitors than the linear ones (BSA-LBP and BSA-BA6).

In line with the results obtained in the above described *in vitro* adhesion assays, exogenously added BSP-peptides were capable of inhibiting breast cancer cell attachment to cryostat sections of trabecular bone (2-day-old neonatal mouse tail vertebrae; Fig. 6). The

susceptibility of tumor cells to various BSP-peptides increased during maturation of trabecular bone (Fig. 6), resulting in a significant decrease in attachment of the breast cancer cells to the mature remodeled bone (the proximal tail vertebrae 1-11; Fig. 6).

Figs. 7-9 depict the the inhibitory effects of exogenously added CNB during attachment of MDA-MB-231 cells to various SFCM (Fig. 7), cortical bone slices (Fig. 8), and vertebrae (Fig. 9). The micrographs show that both adhesion of MDA-MB-231 cells to extracellular bone matrix and subsequent spreading are strongly decreased.

## DISCUSSION

Several extracellular matrix proteins with cell attachment properties have been isolated from skeletal tissue (11, 13, 16-22, 49-51). These include fibronectin, type I collagen, vitronectin, thrombospondin, OPN, and BSP. Bone adhesion proteins are either produced by bone cells and/or derived from extracellular sources and are involved in a variety of functions, such as cellular differentiation and proliferation of bone cells, their migration, and attachment (11-13, 16-19, 23-25, 30, 31, 51). The bone glycoproteins BSP and OPN are differentially expressed during maturation of bone, especially at the onset of calcification (11, 16, 18-25, 31, 49, 52). Furthermore, cells in bone (osteoclasts, osteoblasts, osteocytes, and other stromal cells) can recognize these extracellular molecules or synthetic peptide sequences that encompass the RGD sequence (16, 29, 31-36, 53-55). Evidence is mounting that osteoclasts that highly express the vitronectin receptor  $\alpha_v\beta_3$  use this integrin to adhere to these extracellular bone matrix proteins like BSP, and it has been suggested that that this adhesion receptor is a prerequisite for osteoclastic bone resorption and osteoclast development (29-36).

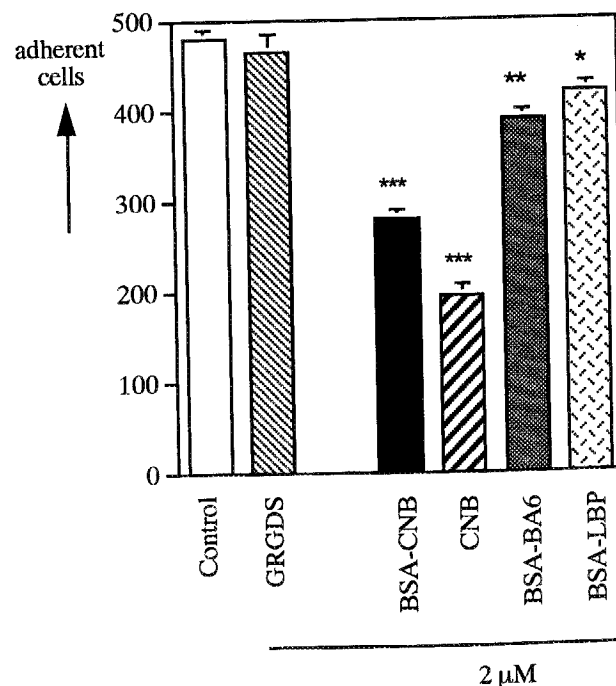
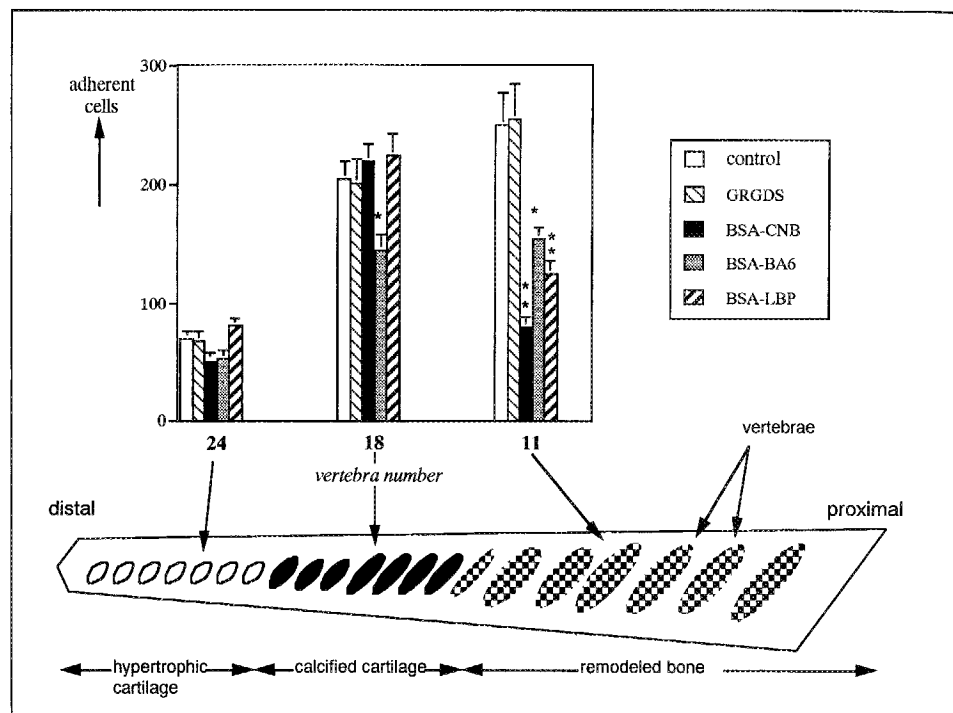


Fig. 5. Inhibitory effects of exogenously added BSP-peptides ( $2 \mu$ M) on adhesion of breast cancer cells (MDA-MB-231) to extracellular bone matrix (bovine cortical bone slices). Two  $\mu$ M GRGDS were used to study the RGD-dependent nature of tumor cell attachment. BSA, GRADS, and GRGES were used as negative controls and did not change adhesion of mammary carcinoma cells to bone matrix (results not shown). Each group was performed in 4-fold, and experiments were repeated three times; bars, SEM. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.005$ ; \*\*\*,  $P \leq 0.0005$ .

Fig. 6. Inhibitory effects of exogenously added BSP-peptides ( $2 \mu\text{M}$ ) on adhesion of breast cancer cells (MDA-MB-231) to extracellular bone matrix (neonatal mouse tail vertebrae). The number of attached cells per area ( $2.54 \text{ mm}^2$ ) on vertebrae 24, 18 and 11, representing hypertrophic cartilage, calcified cartilage, and mature remodeled trabecular bone, respectively, were counted. Two  $\mu\text{M}$  GRGDS was used to study the RGD-dependent nature of tumor cell attachment. A representative experiment is shown. BSA, GRGDS, and GRGES were used as negative controls and did not change adhesion of mammary carcinoma cells to bone matrix (results not shown). Values are expressed as means ( $n = 5$ ). Each experiment was repeated at least three times; bars, SEM. \*,  $P \leq 0.05$ ; \*\*,  $P \leq 0.005$ .



In analogy to osteoclasts, it has been shown that invasive mammary carcinomas express the  $\alpha_v\beta_3$  receptor (37, 39, 40, 42, 43), and it was reported recently that breast cancer cells show a selective increase in  $\beta_3$  integrins in skeletal metastases (56). Furthermore, recent evidence suggests that highly invasive primary breast carcinomas can express high levels of BSP when compared to noninvasive breast cancers and normal breast tissue (57). We have also shown recently that various mammary carcinoma cell lines have high affinities for the BSP molecule *in vitro* (42).<sup>5</sup> We hypothesized, therefore, that mammary carcinoma cells use mechanism(s) of bone recognition that are comparable to those used by normal cells in bone.

In this study, we describe interactions of breast cancer cells with synthetic BSP peptides. Our data show that mammary carcinoma cells (MDA-MB-231) recognize various immobilized (and solubilized) synthetic BSP sequences, which include the RGD motif and its flanking regions. Furthermore, adhesion of breast cancer cells to extracellular matrix of bone was significantly inhibited by nearly all peptides containing (parts of) the EPRGDNYR sequence (BSA-CNB, CNB > Ac-CNB, BSA-LBP > BSA-BA6 > C-BC3). The cyclic peptides BSA-CNB, CNB, and Ac-CNB were generally more potent inhibitors than linear peptides with similar or shortened amino acid sequences (BSA-BA6 and BSA-LBP). This strongly suggests that tertiary structures of the peptides are important for their ability to interfere with tumor cell adhesion to bone matrix. Our observations are in line with earlier publications using different RGD-containing cyclic and linear peptides and other cell types. Several RGD-containing cyclic (and linear) peptides have proven to be strong and selective inhibitors of cellular adhesion *in vitro* (15, 58) and metastasis formation *in vivo* (59) under different experimental conditions. Furthermore, it was shown recently that human trabecular bone cells adhere and spread on immobilized constructs containing the EPRGDNYR motif of BSP (44), which is in agreement with our data on breast cancer cells.

The inhibitory effects of the tested BSP-peptide analogues on tumor

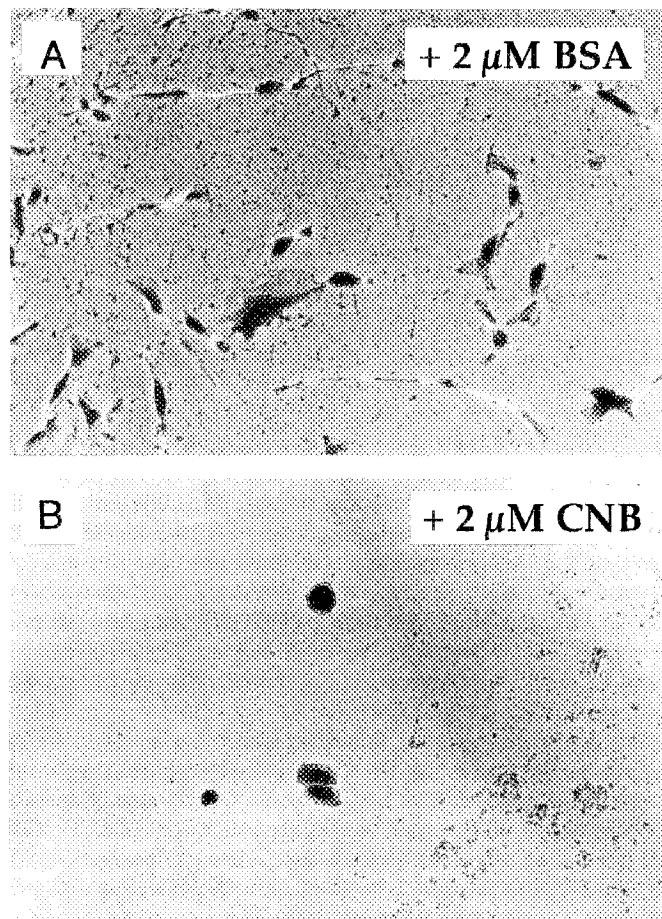


Fig. 7. Micrographs of MDA-MB-231 cells in the absence (A) or presence (B) of  $2 \mu\text{M}$  CNB peptide during 3 h incubation onto SFCM of human trabecular bone cells coated onto plastic.  $\times 200$ .

<sup>5</sup> G. van der Pluijm, H. Vloedgraven, S. Papapoulos, C. Löwik, L. van der Wee-Pals, W. Grzesik, J. Kerr, and P. Gehron Robey, manuscript in preparation.



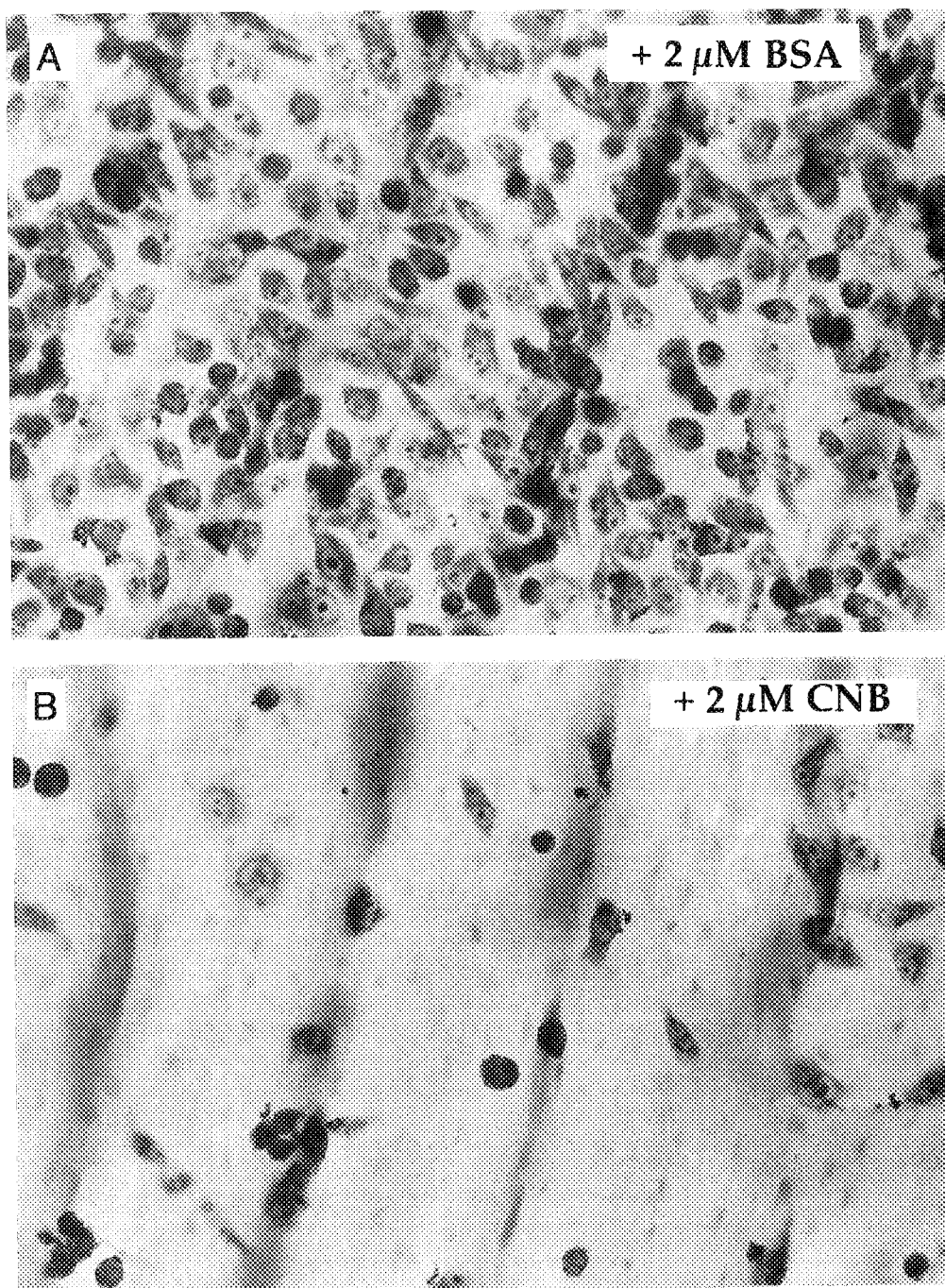


Fig. 8. Micrographs of MDA-MB-231 cells in the absence or presence of 2  $\mu$ M CNB peptide during 3 h incubation on bovine cortical bone slices (B). A, the negative control (2  $\mu$ M BSA).  $\times 200$ .

cell adhesion to bone, together with the lack of an effect of the synthetic non-BSP-derived RGD-peptides GRGDS and C-CB1 (up to 100-fold higher concentrations) indicate that the RGD sequence by itself is not the sole prerequisite for blocking breast cancer cell adhesion to bone matrix under these experimental conditions. Modification of the  $\text{NH}_2$ - and  $\text{COOH}$ -terminal ends of the EPRGDNYR sequence (see Ac-CNB *versus* CNB and BSA-BA6 *versus* BSA-LBP) negatively affected the inhibitory potency of the peptides. This suggests that the presence of the  $\text{NH}_2$ -terminal cationic and  $\text{COOH}$ -terminal anionic groups can modify the functional response mediated by its receptor(s), as has been reported earlier for osteoclasts (60).

Furthermore, our data support the notion that RGD flanking regions of BSP (EP-RGD-NYR), which differ from the more common RGD recognition sequence of fibronectin (G-RGD-SPC), may additionally determine the inhibitory potency of the peptides

on breast cancer cell adhesion to bone matrix. Therefore, proper positioning of the RGD motif within the framework of its BSP-flanking regions strongly enhances the inhibitory potency of the synthetic peptides on breast cancer cell adhesion to bone matrix.

In conclusion, we have demonstrated that breast cancer cells recognize synthetic peptides encompassing the RGD motif of human BSP in various conformations. Attachment of cancer cells to three *in vitro* models for extracellular matrix of bone (serum-conditioned medium of bone cells, cortical bone slices, and frozen sections of trabecular bone) was effectively inhibited by exogenously added BSP peptides. This inhibition is most likely mediated through adhesion receptors of the integrin family that recognize RGD motifs and/or its flanking regions. Clearly, further studies are warranted to establish the relevance of these observa-

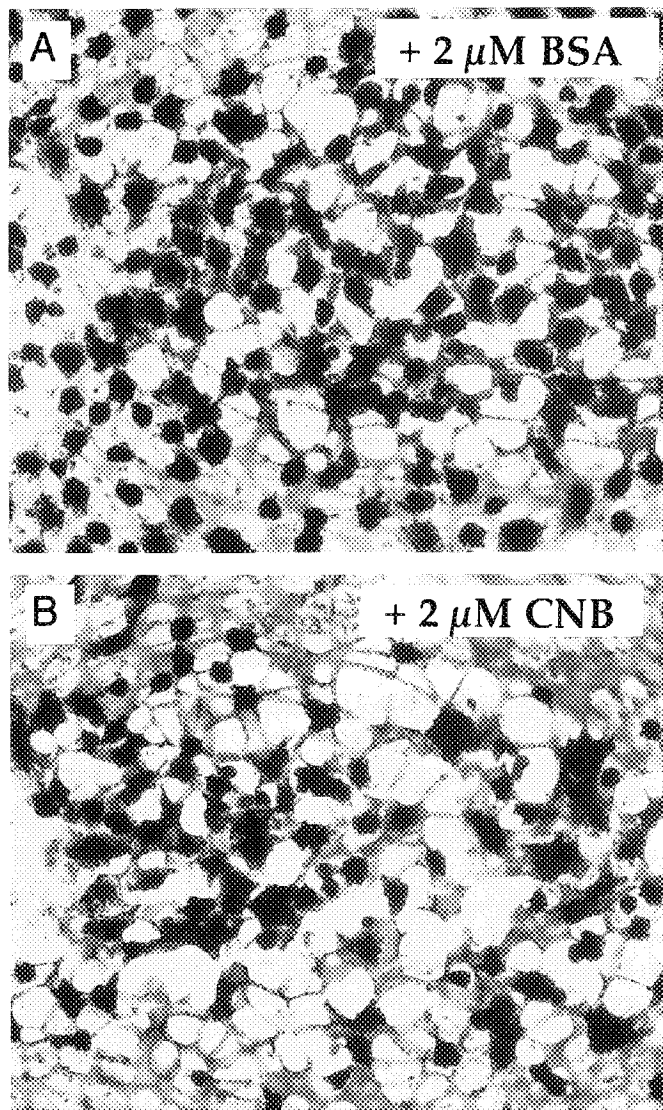


Fig. 9. Micrographs of MDA-MB-231 cells in the absence or presence of 2  $\mu$ M CNB peptide during 3 h incubation on frozen sections of mouse tail vertebrae-11 (B). A, the negative control (2  $\mu$ M BSA).  $\times 200$ .

tions in the development and treatment of metastatic bone disease *in vivo*.

## REFERENCES

- Elte, J. W. F., Bijvoet, O. L. M., Clifton, F. J., van Oosterom, A. T., and Sleeboom, H. P. Osteolytic metastases in breast carcinoma: pathogenesis, morbidity and bisphosphonate treatment. *Eur. J. Cancer Clin. Oncol.*, 22: 493-500, 1986.
- Paterson, A. H. G. Bone metastases in breast cancer, prostate cancer and myeloma. *Bone*, 8 (Suppl. 1): S17-S22, 1987.
- Coleman, R. E., and Rubens, R. D. The clinical course of bone metastases from breast cancer. *Br. J. Cancer*, 55: 61-66, 1987.
- Kanis, J. A., Gray, R. E., Urwin, G., Murray, S., and Hamdy, N. Physiology of bone and metabolic approaches to the treatment of skeletal metastases. *Dev. Oncol.*, 51: 303-322, 1987.
- Berrettoni, B. A., and Carter, J. R. Mechanisms of cancer metastasis to bone. *J. Bone Jt. Surg. Am. Vol.*, 68: 308-312, 1986.
- Rosol, T. J., and Capen, C. C. Biology of disease: mechanisms of cancer-induced hypercalcemia. *Lab. Invest.*, 67: 680-702, 1992.
- Orr, F. W., Kostenuik, P., Sanchez-Sweetman, O. H., and Singh, G. Mechanisms involved in the metastasis of cancer to bone. *Breast Cancer Res. Treat.*, 25: 151-163, 1993.
- Yoneda, T., Sasaki, A., and Mundy, G. R. Osteolytic bone disease in breast cancer. *Breast Cancer Res. Treat.*, 32: 73-84, 1994.
- Maemura, M., and Dickson, R. B. Are cellular adhesion molecules involved in the metastasis of breast cancer? *Breast Cancer Res. Treat.*, 32: 239-260, 1994.
- Lochter, A., and Bissell, M. J. Involvement of extracellular matrix constituents in breast cancer. *Semin. Cancer Biol.*, 6: 165-173, 1995.
- Gehron Robey, P. The biochemistry of bone (Review). *Endocrinol. Metab. Clin. North Am.*, 18: 859-902, 1989.
- Butler, W. T. Sialoproteins of bone and dentin (Review). *J. Biol. Buccale*, 19: 83-89, 1991.
- Termine, J. D. Bone matrix proteins and the mineralization process. In: M. J. Favus (ed.), *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*, pp. 21-25. New York: Raven Press, 1993.
- Hynes, R. O. Integrins: versatility, modulation and signaling in cell adhesion. *Cell*, 69: 11-25, 1992.
- Pierschbacher, M. D., and Ruoslahti, E. Influence of stereochemistry of the sequence Arg-Gly-Asp-Xaa on binding specificity in cell adhesion. *J. Biol. Chem.*, 262: 17924-17928, 1987.
- Grzesik, W. J., and Gehron Robey, P. Bone matrix RGD glycoproteins: immunolocalization and interaction with human primary osteoblastic bone cells *in vitro*. *J. Bone Miner. Res.*, 9: 487-496, 1994.
- Ingram, R. T., Clarke, B. L., Fisher, L. W., and Fitzpatrick, L. A. Distribution of noncollagenous proteins in the matrix of adult human bone: evidence of anatomic and functional heterogeneity. *J. Bone Miner. Res.*, 8: 1019-1029, 1993.
- Bianco, P., Fisher, L. W., Young, M. F., Termine, J. D., and Gehron Robey, P. Expression of bone sialoprotein (BSP) in developing human tissues. *Calcif. Tissue Int.*, 49: 421-426, 1991.
- Fisher, L. W., Whitson, S. W., Avioli, L. V., and Termine, J. D. Matrix sialoprotein of developing bone. *J. Biol. Chem.*, 258: 12723-12727, 1983.
- Hultenby, K., Reinholt, F. P., Norgard, M., Oldberg, A., Wendel, M., and Heinegård, D. Distribution and synthesis of bone sialoprotein in metaphyseal bone of young rats show a distinctly different pattern from that of osteopontin. *Eur. J. Cell Biol.*, 63: 230-239, 1994.
- Chen, J., McKee, M. D., Nanci, A., and Sodek, J. Bone sialoprotein mRNA expression and ultrastructural localization in fetal porcine calvarial bone: comparisons with osteopontin. *Histochem. J.*, 26: 67-78, 1994.
- Bianco, P., Riminucci, M., Silvestrini, G., Bonucci, E., Termine, J. D., Fisher, L. W., and Gehron Robey, P. Localization of bone sialoprotein (BSP) to Golgi and post-Golgi secretory structures in osteoblasts and to discrete sites in early bone matrix. *J. Histochem. Cytochem.*, 41: 193-203, 1993.
- Bianco, P., Riminucci, M., Bonucci, E., Termine, J. D., and Gehron Robey, P. Bone sialoprotein (BSP) secretion and osteoblast differentiation: relationship to bromodeoxyuridine incorporation, alkaline phosphatase, and matrix deposition. *J. Histochem. Cytochem.*, 41: 183-191, 1993.
- Yao, K. L., Todescan, R., Jr., and Sodek, J. Temporal changes in matrix protein synthesis and mRNA expression during mineralized tissue formation by adult rat bone marrow cells in culture. *J. Bone Miner. Res.*, 9: 231-240, 1994.
- Ibaraki, K., Termine, J. D., Whitson, S. W., and Young, M. F. Bone matrix mRNA expression in differentiating fetal bovine osteoblasts. *J. Bone Miner. Res.*, 7: 743-754, 1992.
- Chen, J., Shapiro, H. S., and Sodek, J. Developmental expression of bone sialoprotein mRNA in rat mineralized connective tissues. *J. Bone Miner. Res.*, 7: 987-997, 1992.
- Chenu, C., Colucci, S., Grano, M., Zigrino, P., Barattolo, R., Zamboni, G., Baldini, N., Vergnaud, P., Delmas, P. D., and Zallone, A. Z. Osteocalcin induces chemotaxis, secretion of matrix proteins, and calcium-mediated intracellular signaling in human osteoclast-like cells. *J. Cell Biol.*, 127: 1149-1158, 1994.
- Beresford, J. N., Joyner, C. J., Devlin, C., and Triffitt, J. T. The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells *in vitro*. *Arch. Oral Biol.*, 39: 941-947, 1994.
- Reinholt, F. P., Hultenby, K., Oldberg, A., and Heinegård, D. Osteopontin: a possible anchor of osteoclasts to bone. *Proc. Natl. Acad. Sci. USA*, 87: 4473-4475, 1990.
- Oldberg, A., Franzen, A., and Heinegård, D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell binding sequence. *Proc. Natl. Acad. Sci. USA*, 83: 8819-8823, 1986.
- Chen, J., Singh, K., Mukherjee, B. B., and Sodek, J. Developmental expression of osteopontin (OPN) mRNA in rat tissues: evidence for a role for OPN in bone formation and resorption. *Matrix*, 13: 113-123, 1993.
- Helfrich, M. H., Nesbitt, S. A., Dorcy, E. L., and Horton, M. A. Rat osteoclasts adhere to a wide range of RGD (Arg-Gly-Asp) peptide-containing proteins, including the bone sialoproteins and fibronectin, via a  $\beta 3$  integrin. *J. Bone Miner. Res.*, 7: 335-343, 1992.
- Ek-Rylander, B., Flores, M., Wendel, M., Heinegård, D., and Andersson, G. Dephosphorylation of osteopontin and bone sialoprotein by osteoclastic tartrate-resistant acid phosphatase. Modulation of osteoclast adhesion *in vitro*. *J. Biol. Chem.*, 269: 14853-14856, 1994.
- Davies, J., Warwick, J., Totty, N., Philip, R., Helfrich, M., and Horton, M. The osteoclast functional antigen, implicated in the regulation of bone resorption, is biochemically related to the vitronectin receptor. *J. Cell Biol.*, 109: 1817-1826, 1989.
- Ross, F. P., Chappel, J., Alvarez, J. I., Sander, D., Butler, W. T., Farach-Carson, M. C., Mintz, K. A., Robey, P. G., Teitelbaum, S. L., and Cheresch, D. A. Interactions between bone matrix proteins osteopontin and bone sialoprotein and the osteoclast integrin  $\alpha v \beta 3$  potentiate bone resorption. *J. Biol. Chem.*, 268: 9901-9907, 1993.
- van der Pluijm, G., Mouthaan, H., Baas, C., de Groot, H., Papapoulos, S., and Löwik, C. Integrins and osteoclastic resorption in three bone organ cultures: differential sensitivity to synthetic Arg-Gly-Asp peptides during osteoclast formation. *J. Bone Miner. Res.*, 9: 1021-1028, 1994.
- Koukoulis, G. K., Virtanen, I., Korhonen, M., Laitinen, L., Quaranta, V., and Gould, V. E. Immunohistochemical localization of integrins in normal, hyperplastic, and neoplastic breast. *Am. J. Pathol.*, 139: 787-799, 1991.
- Juliano, R. L. The role of  $\beta_1$  integrins in tumors. *Semin. Cancer Biol.*, 4: 277-283, 1993.

39. Bernstein, L. R., and Liotta, L. A. Molecular mediators of interactions with extracellular matrix components in metastasis and angiogenesis. *Curr. Opin. Oncol.*, 6: 106-113, 1994.
40. Pignatelli, M., Cardillo, M. R., Hanby, A., and Stamp, G. W. Integrins and their accessory adhesion molecules in mammary carcinomas: loss of polarization in poorly differentiated tumors. *Hum. Pathol.*, 23: 1159-1166, 1992.
41. Zetter, B. R. Adhesion molecules in tumor metastasis. *Semin. Cancer Biol.*, 4: 219-229, 1993.
42. van der Pluijm, G., Kerr, J., Löwik, C., and Gehron Robey, P.  $\beta 1$  and  $\beta 3$  integrin subunits are involved in adhesion of breast cancer cells to extracellular bone matrix. *J. Bone Miner. Res.*, 8 (Suppl.): S136, 1993.
43. van der Pluijm, G., Vloedgraven, H., Papapoulos, S., Gehron Robey, P., and Löwik, C. Integrins mediate adhesion of breast cancer cells to various stages of developing bone. *J. Bone Miner. Res.*, 9 (Suppl.): S421, 1994.
44. Ivanov, B., Grzesik, W., and Robey, F. A. Synthesis and use of a new bromoacetyl-derivatized heterotrifunctional amino acid for conjugation of cyclic RGD-containing peptides derived from human bone sialoprotein. *Bioconjugate Chem.*, 6: 269-277, 1995.
45. Cailleau, R., Young, R., Olivé, M., and Reeves, W. J. Breast tumor cell lines from pleural effusions. *J. Natl. Cancer Inst.*, 53: 661-674, 1974.
46. Sasaki, A., Boyce, B. F., Story, B., Wright, K. R., Chapman, M., Boyce, R., Mundy, G. R., and Yoneda, T. Bisphosphonate risedronate reduces metastatic human breast cancer burden in bone in nude mice. *Cancer Res.*, 55: 3551-3557, 1995.
47. Gehron Robey, P., and Termine, J. D. Human bone cells *in vitro*. *Calcif. Tissue Int.*, 37: 453-460, 1985.
48. Barker, L., Bullens, S., Bunting, S., Burdick, D. J., Chan, K. S., Deisher, T., Eigenbrot, C., Gadec, T. R., Gantz, R., Lipari, M. T., Muir, C. D., Napier, M. A., Pitti, R. M., Padua, A., Quan, C., Stanley, M., Struble, M., Tom, J. Y. K., and Burnier, J. P. Cyclic RGD peptide analogues as antiplatelet antithrombotics. *J. Med. Chem.*, 35: 2040-2048, 1992.
49. Fisher, L. W. Structure/function studies of the sialoglycoproteins and proteoglycans of bone: it is still the early days. In: H. Slavkin and P. Price (eds.), *Chemistry and Biology of Mineralized Tissues*, pp. 177-186. Amsterdam: Elsevier Science Publishers, B. V., 1992.
50. Oldberg, A., Franzen, A., and Heinegård, D. The primary structure of a cell binding sialoprotein. *J. Biol. Chem.*, 263: 19430-19432, 1988.
51. Oldberg, A., Franzen, A., and Heinegård, D. Cloning and sequence analysis of rat bone sialoprotein (osteopontin) cDNA reveals an Arg-Gly-Asp cell binding sequence. *Proc. Natl. Acad. Sci. USA*, 83: 8819-8823, 1986.
52. Hunter, G. K., and Goldberg, H. A. Nucleation of hydroxyapatite by bone sialoprotein. *Proc. Natl. Acad. Sci. USA*, 90: 8562-8565, 1993.
53. Paniccia, R., Colucci, S., Grano, M., Serra, M., Zallone, A. Z., and Teti, A. Immediate cell signal by bone-related peptides in human osteoclast-like cells. *Am. J. Physiol.*, 265: C1289-C1297, 1993.
54. Shankar, G., Davison, I., Helfrich, M. H., Mason, W. T., and Horton, M. A. Integrin receptor-mediated mobilisation of intranuclear calcium in rat osteoclasts. *J. Cell Sci.*, 105: 61-68, 1993.
55. Clark, E. A., and Brugge, J. S. Integrins and signal transduction pathways: the road taken. *Science (Washington DC)*, 268: 233-239, 1995.
56. Kitazawa, S., and Macda, S. Development of skeletal metastases. *Clin. Orthop. Relat. Res.*, 312: 45-50, 1995.
57. Bellahcene, A., Merville, M., and Castronovo, V. Expression of bone sialoprotein, a bone matrix protein, in human breast cancer. *Cancer Res.*, 54: 2823-2826, 1994.
58. Aumailley, M., Gurrath, M., Müller, G., Calvete, J., Timpl, R., and Kessler, H. Arg-Gly-Asp constrained within cyclic pentapeptides: strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1. *FEBS Lett.*, 291: 50-54, 1991.
59. Kumagai, H., Tajima, M., Ueno, Y., Giga-Hama, Y., and Ohba, M. Effect of cyclic RGD peptide on cell adhesion and tumor metastasis. *Biochem. Biophys. Res. Commun.*, 177: 74-82, 1991.
60. Horton, M. A., Dorey, E. L., Nesbitt, S. A., Samanen, J., Ali, F. E., Stadel, J. M., Nichols, A., Grieg, R., and Helfrich, M. H. Modulation of vitronectin receptor-mediated osteoclast adhesion by Arg-Gly-Asp peptide analogs: a structure-function analysis. *J. Bone Miner. Res.*, 8: 239-247, 1993.